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Exhibit 4

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Genes encoding a mouse monoclonal antibody are expressed in transgenic mice, rabbits and pigs

(Recombinant DNA; transgenic animals; detection of transgenes; antibody analytics)

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SUMMARY

To study the expression pattern of immunoglobulin-encoding genes in transgenic animals, we have introduced the genes for the light and heavy chain of a mouse monoclonal antibody (mAb) into the germ-line of mice (control), rabbits and pigs. The transgenes were detected in the mouse lines, two rabbit lines and pigs. Titers of 100–200 μg mAb/ml (rabbits) and up to 1000 μg mAb/ml (pig) were measured in the sera of the transgenic animals. Isoelectric focusing experiments with serum followed by immunofixation revealed that in the transgenic pig only a minority of the bands were identical to those of the purified mouse mAb. In transgenic rabbits we found no coincidence of bands at all. The results can be explained by assuming tissue- and cell-type-specific glycosylation, modification and possible heterologous chain associations. Expression of Ab in the serum of animals could help to protect against diseases (e.g., influenza in pigs).

INTRODUCTION

Expression of cloned genes of mAb has been extensively investigated in transgenic mice (for review: Storb, 1987). These mice expressed large amounts of Ab to nitrophenyl, trinitrophenyl and phosphorylcholine without prior immunisation (Grosschedl et al., 1984; Rusconi and Köhler, 1985; Storb et al., 1986). These studies demonstrated that the rearrangement of endogenous immunoglobulin genes can be inhibited by transgenes (Ritchie et al., 1984; Weaver et al., 1985; Nussenzweig et al., 1987). The studies cited above primarily focused on events induced by a transgene at the genomic level. In most of the studies only genes for single chains were introduced into the germ-line of mice and therefore the exact composition of the secreted Ab was of marginal importance. In an attempt to extend these studies to other animals we have introduced genes for the light and heavy chain of a mouse mAb into the germ-line of mice, as a control, and also into the germ-line of rabbits and pigs. The experiments were designed to evaluate whether Ab of diagnostic and therapeutic interest could be produced in large amounts in the serum of these animals. Furthermore, expression of Ab genes in these species would be a first step towards in vivo immunisation against bacterial and viral diseases.

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Abbreviations: Ab, antibody(ies); ABTS, 2,2′-azino-di(3-ethylbenzthiazoline-sulfonate-6); bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; Fab fragments, antigen-binding fragments of Ab prepared by papain digestion that contain the light chain and part of the heavy chain (variable region and first constant domain); FACS, fluorescence-activated cell sorting; Fc, antibody fragment prepared by digestion with papain and consisting of the second and third domain of the constant region of the γ-chain; γ1 chain, mouse immunoglobulin heavy γ1-chain; HCG, human chorion gonadotropin; Hepes, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid; IEF, isoelectric focusing; Ig, immunoglobulin; I.U., international units; kb, kilobase(s) or 1000 bp; mAb, monoclonal Ab; mAb A20/40, monoclonal anti-idiotypic antibody directed against mAb-NP; mAb-NP, mAb directed against NP; NP, 4-hydroxy-3-nitrophenylacetate; nt, nucleotide(s); PMSG, pregnant mare serum gonadotropin; POD, peroxidase.

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RESULTS AND DISCUSSION

(a) The Ab-encoding genes used for introduction into the germ-line

The Ig heavy- and light-chain genes (encoding a γ-1 and a κ chain) were isolated from the IgG1 secreting hybridoma cell line A20/44 (Sablitzky et al., 1985a,b; Kocks and Rajewsky, 1988). The Ab secreted from this hybridoma cell line will be referred to as mAb A20/44, an anti-idiotypic Ab directed against mAb-NP which was isolated from the hybridoma cell line S43 (Reth et al., 1978). mAb-NP is directed against the hapten 4-hydroxy-3-nitro-phenylacetate. As model genes for the introduction into the germ-line we have used the κ-encoding gene (5.5 kb) and the γ-1-encoding gene (9.25 kb) of mAb A20/44.

The genes were subcloned into a prokaryotic vector in a head-to-tail and a head-to-head fashion (Lenz and Weidle, 1990). After gene transfer by electroporation into the non-producer hybridoma cell line Sp2/0 (Ochi et al., 1983) permanent cell lines secreting reconstituted Ab could be established (data not shown). The two different gene configurations were equally well expressed after gene transfer into Sp2/0. After deletion of the *Sal*I site between the κ and γ-1-encoding genes the genes could be isolated as *Sal*I fragments free of prokaryotic sequences and used for microinjection into the pronucleus of fertilized eggs. The fragments used for microinjection as well as the probes used for the Southern blotting experiments are shown in Fig. 1.

(b) Synthesis of reconstituted Ab in the serum of transgenic mice, rabbits and pigs

The serum levels of reconstituted Ab A20/44 of founder animals and their offspring were quantified by ELISA (Fig. 2). In this assay there was no cross-reactivity with sera from control mice, rabbits and pigs. It must be noted that this procedure gives a measure of the specific antigen-binding activity but does not yield information about the exact composition of the Ab (i.e., it does not allow the distribution of homo-dimeric and hetero-dimeric Ab complexes).

Ab A20/44 synthesis in two mouse lines (970-08 with 20 μg/ml and 974-10 with 38 μg/ml) is displayed in Fig. 2, A and B. The reason for the markedly increased Ab level in the serum of some offspring of mouse 974-10 is not clear. Thirty rabbits were derived after microinjection of the Ig genes into the pronucleus of fertilized eggs. The sera of two of them scored positive in the ELISA test (animal 2644 with 300 μg/ml and animal 2665 with 140 μg/ml). The A20/44-related activity in the serum of positive transgenic lines is shown in Fig. 2C.

The serum of one out of three pigs scored positive for Ab A20/44 with a surprisingly high titer (1 mg/ml; Fig. 2D). This titer could be confirmed ($\pm$ 200 μg/ml) in six independent blood samples derived during six months of observation. The differences in Ab titers in different species cannot be explained by different total serum Ig titers, because the levels were comparable (0.25 g/100 ml). The γ1 transgene

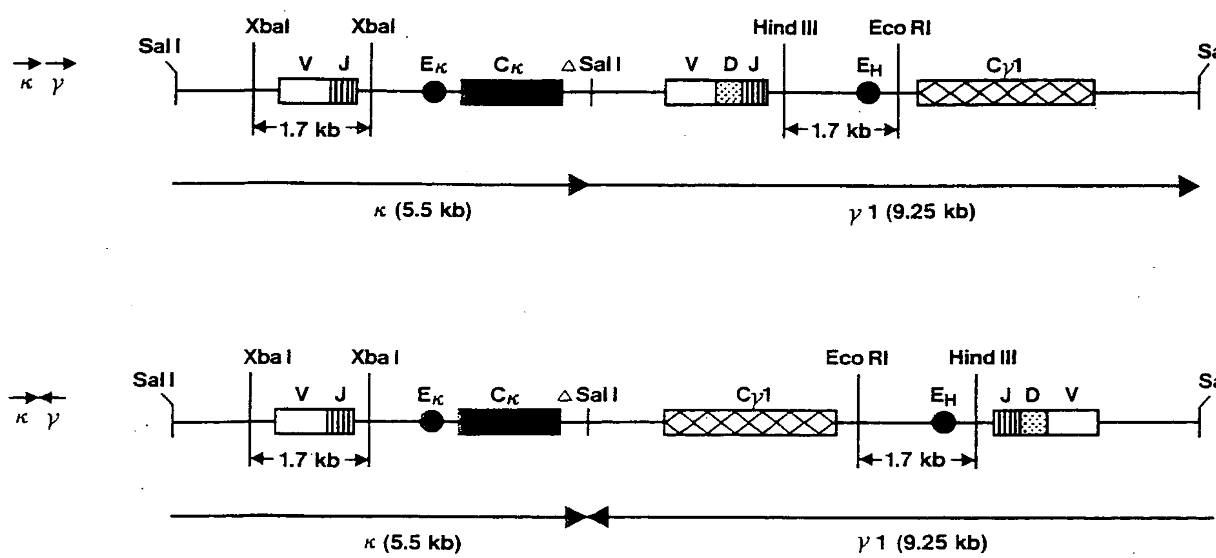

Fig. 1. Fragments used for microinjection and for Southern blotting experiments. The *Sal*I fragments (with head-to-tail and head-to-head configuration of the genes for the light and heavy chain of mAb A20/44) were isolated on a low-melting agarose gel and used for microinjection into the pronucleus of fertilized eggs. The κ and γ-1-encoding genes are indicated by arrows. $\overrightarrow{\kappa\gamma}$ and $\overrightarrow{\kappa}\overleftarrow{\gamma}$ indicate the different orientations of the γ-1 gene with respect to κ. E_κ and E_H, enhancers for mouse light and heavy Ig-encoding genes; J, joining regions of mouse Ig-encoding genes; D, diversity region of mouse heavy-chain-encoding gene; C_κ and $C_\gamma 1$, constant regions of mouse light and heavy Ig-encoding genes. For Southern blotting the indicated 1.7-kb *Xba*I fragment (κ chain) and 1.7-kb *Eco*RI-*Hin*dIII fragment (γ-1 chain) were used.

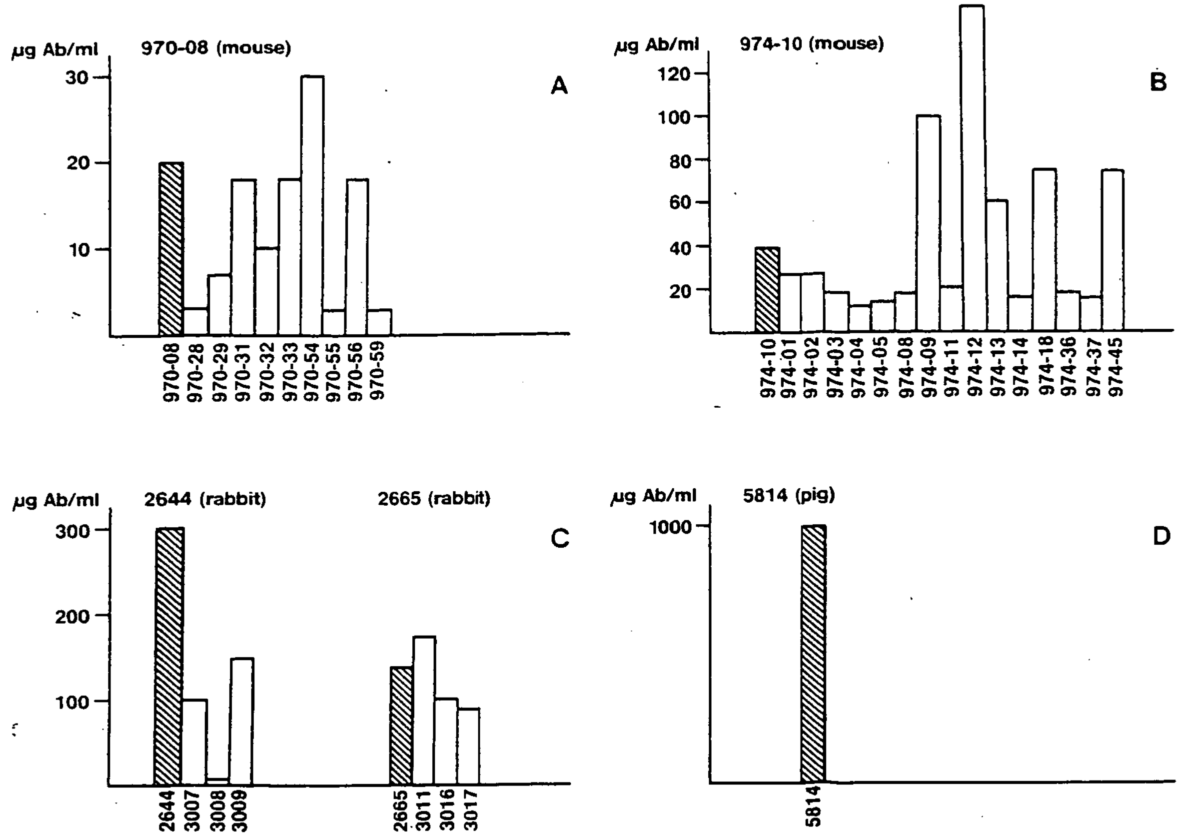

Fig. 2. Expression of reconstituted Ab in the serum of transgenic mice, rabbits and pigs. Production of transgenic mice, rabbits and pigs was carried out as described previously (Brem et al., 1985). **Mice.** Oocytes were collected from superovulated (10 I.U. PMSG, 10 I.U. HCG two days later) NMRI outbred mice. Injected zygotes were transferred to foster mothers of the same strain. **Rabbits.** Hybrid rabbits (ZIKA^R) were superovulated using 150 I.U. PMSG and before mating induction of ovulation was achieved by application of 180 I.U. HCG in donor and recipient animals. 20 h after insemination oocytes were collected, injected and transferred into foster mothers. **Pigs.** Oocytes were collected from superovulated (1250 I.U. PMSG, 600 I.U. HCG) landrace pigs 60 h after injection of HCG. Oocytes were centrifuged at 15 000 × *g* for 3 min to facilitate the visualisation of the pronuclei. About 40 injected one-cell eggs were transferred to one oviduct of each recipient. **Microinjection.** Microinjection was carried out under multi 400 × magnification using a ZEISS Inverted Microscope ICM 405 and Nomarski interference contrast optics. Zygotes were placed on a depression slide in a drop of flushing medium. Zygotes were held on a holding pipette by suction, and the injection pipette (diameter about 1 µm) was inserted into one of the pronuclei. Injection was done by air pressure. The expansion of the pronuclei demonstrated the successful injection of several picoliters of DNA solution including about 1000 copies of the gene construct. Serum levels of A20/44 were quantitated by ELISA. Microtiter plates were coated with mAb NP (10 µg/ml) in 0.2 M carbonate buffer pH 9.4, for 2 h. The wells were then incubated with 50 mM Hepes pH 7.0/0.15 M NaCl/1% crotein C (gelatin hydrolysate) for 30 min and the buffer was carefully removed. The plates were incubated with various dilutions of the sera for 2 h at 25°C. After washing with 50 mM Hepes pH 7.0/0.15 mM NaCl/0.2 M Na_2·tartrate/1% crotein C/0.75% pluronic F68/0.01% phenol, the samples were incubated with POD-conjugated Fab fragments of mAb NP (159 mU POD/ml). After washing with the buffer described above, POD was quantified with hydrogen peroxide and ABTS and the A_{405nm} determined by an ELISA reader. Purified mAb A20/44 was used as a standard. Hatched bars represent the founder animal, open bars represent offspring, with their Nos. stated underneath.

does not contain the membrane exons. Transgene expression on the surface of lymphocytes could not be detected by FACS analysis.

(c) Detection of the transgenes by Southern blotting with genomic DNA

DNA from transgenic animals is expected to contain an indicative 1.7-kb *Xba*I fragment for the κ chain and a 1.7-kb *Eco*RI-*Hin*dIII fragment specific for the γ1-chain. High-M_r DNA was isolated from control and transgenic animals, digested with *Xba*I or with *Eco*RI + *Hin*dIII and hybridized against the fragments as shown in Fig. 1. Mice 970-08 and 974-10 as well as rabbits 2644 and 2655 are derived from construct $\overline{\kappa\gamma}$, pig 5814 is derived from construct $\overline{\kappa\gamma}$.

The 1.7-kb fragment was detected in the genome of the founder mice 970-08 and 974-10 and some of their offspring (Fig. 3C), in the genome of the transgenic rabbits 2644 and 2665 (Fig. 3C) and in the genome of the transgenic pig 5814 (Fig. 3D). It was consistently absent in the genome of control mice, rabbits and pigs. By comparing the intensities of

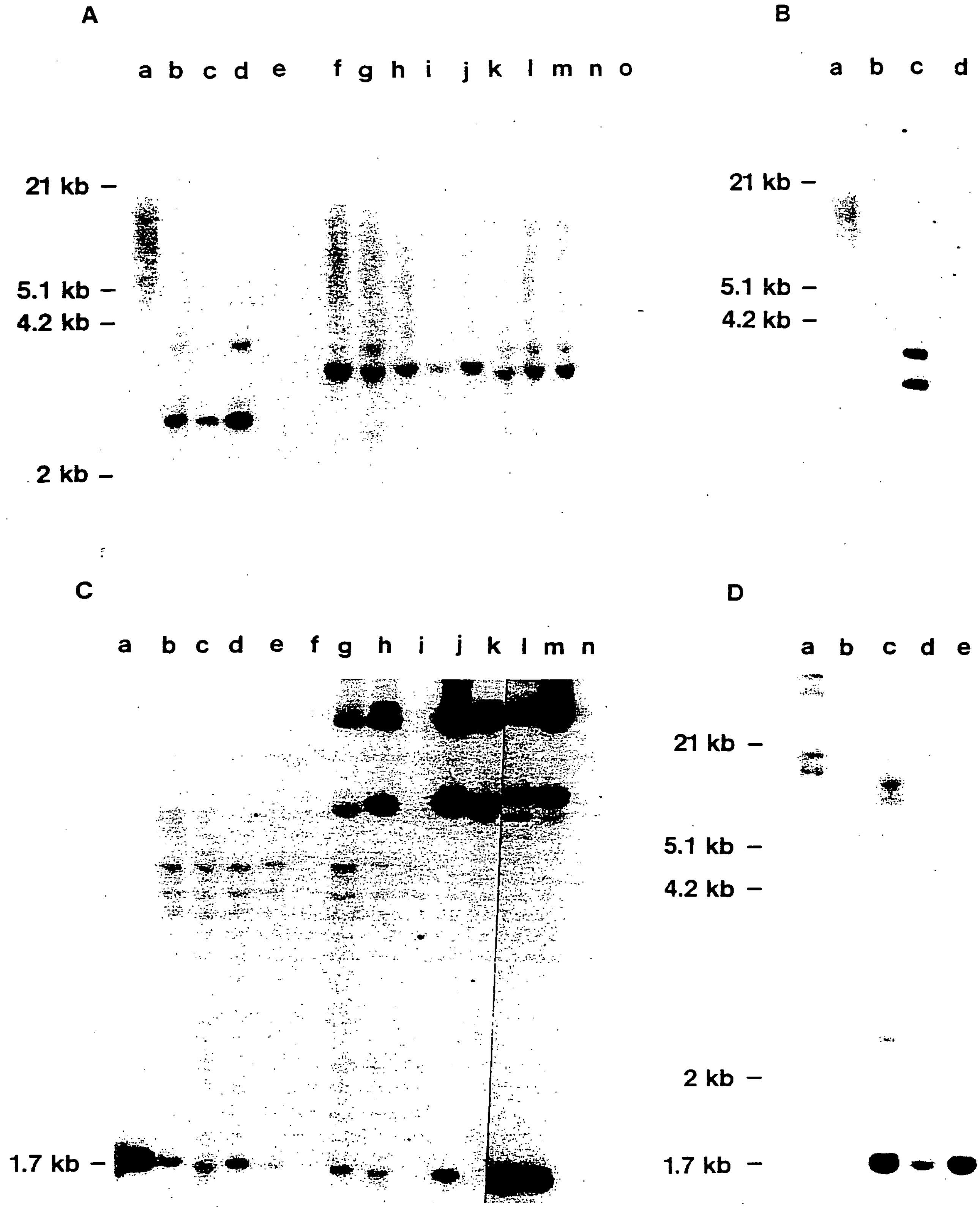

Fig. 3. Detection of transgenes by Southern blotting. DNA was extracted from mouse tail tissue, from blood cells of rabbits and from blood cells and ear tissue of pigs. A 1.7-kb *Xba*I-fragment was used as a probe to detect the mouse light chain (see Fig. 1). The fragment includes parts of the 5′-untranslated region and the VJ region of the mouse κ chain. A 1.7-kb *Eco*RI-*Hin*dIII fragment was used as a probe to detect the mouse heavy chain (see Fig. 1). The fragment includes intron sequences of the mouse heavy chain. The DNA (20 μg unless indicated otherwise) was cleaved with the

the diagnostic band with that from the hybridoma cell line A20/44 we estimated about 50 copies of the transgene in pig 5814. A similar analysis for the transgenic rabbits 2644 and 2665 indicated about 40–60 copies of the transgene in their genomes (data not shown). For the transgenic mice, the intensity of the relevant fragments corresponds to one to three copies of transgene per genome. Using a light-chain probe no hybridisation to control rabbit and pig DNA was observed. The transgenic status of rabbits 2644 and 2665 and pig 5814 was confirmed (Fig. 3, A and B). Mice derived from founder animal 970-08 or 974-10 differ slightly in their hybridisation pattern (Fig. 3A). We observed a diffuse signal with DNA from control mice and the hybridoma cell line A20/44, due to cross-hybridisation with other light-chain fragments. We do not know yet, whether pig 5814 is transmitting the transgene to its offspring (transgenic or mosaic status). We do not have an explanation for the high-M_r bands seen in Fig. 3C.

(d) Characterisation of the Ab expressed in the serum of transgenic rabbits and pigs

Sera from control and transgenic animals were investigated by IEF and subsequent immunofixation by incubation with anti Fcγ serum and silver staining of the complexes. The sera of the mice could not be analysed this way, due to cross-reactivity. Surprisingly, only a minor portion of the immunoreactive material in the serum of the transgenic pig 5814 migrated to positions in the gel corresponding to those of Ab A20/44 purified from ascites fluid using two different pH gradients (Fig. 4A). Upon further dilution of the serum a plethora of immuno-reactive bands could be resolved and the bands corresponding to Ab A20/44 became undetectable (Fig. 4B). At the dilutions used in these experiments no cross-reactivity with serum from control pigs was observed (Fig. 4, A and B). Technical problems could be ruled out by mixing a defined amount of purified Ab 20/44 with control serum and serum from animal 5814. The Ab added to the sera gave rise to the same IEF pattern as the purified Ab (data not shown).

Analysis of control rabbit sera did not reveal any cross-reactivity with our Ab used for immunofixation in the range of dilutions used in these experiments (Fig. 4, C and D). Sera from animals 2644 and 2665 did not show any bands coinciding with those of purified Ab A20/44 (Fig. 4, C and D). Addition of purified Ab A20/44 to sera from control and transgenic rabbits clearly led to the identification of its characteristic IEF bands, thus excluding experimental artifacts (data not shown).

For purification the serum was subjected to $(NH_4)_2SO_4$ precipitation, chromatography on DEAE-Sepharose and absorption to an affinity matrix conjugated with the idiotypic Ab. The purified preparation displayed identical discrete bands as the crude serum by IEF analysis and subsequent immunofixation with goat anti-mouse Fcγ serum (Fig. 4E), but as expected different from those of Ab20/44 purified from ascites fluid. We note that the smear (which is not due to unspecific cross-reactivity) detected with crude serum was absent in the purified preparation (Fig. 4E, lanes a and b). Analysis by ELISA revealed that all of the purified protein had two intact binding sites for the antigen (idiotypic Ab). Our ELISA system only gives rise to a positive signal when two intact binding sites are present.

(e) Conclusion

Protein with antigen-binding activity can be produced in abundant amounts in transgenic rabbits, pigs, and mice when the genes for the κ and γ1 chains of a mouse mAb are introduced into the germ-line of those animals. IEF analysis of the sera has revealed that in the transgenic pig only a minority of the Ab molecules correspond exactly to those of purified mAb20/44 (from ascites), in the sera of the transgenic rabbits no IEF bands coinciding with those of mAb 20/44 were observed. Purification of the Ab from pig serum revealed the presence of Ab with two intact binding sites. The existence of the same discrete bands (IEF analysis) in the serum fraction and the purified Ab preparation from pig serum argues against an association of heterologous chains with those of the mouse at least in the purified preparation. The smear present in crude serum (after immuno-fixation) might be indicative for heterologous Ab by association of the light chains of the rabbit and the pig with the heavy chain of the mouse. The level of mouse κ-chain expression might be insufficient for complete allelic exclusion. In this context it would be interesting to include

◄ appropriate restriction nucleases and separated on a 0.8% agarose gel containing 0.5 μg/ml of ethidium bromide. The DNA was transferred to a nitrocellulose membrane and hybridized with 10^7 cpm of nick-translated probe (Maniatis et al., 1982). Probes: mouse light chain in panels A and B; mouse heavy chain in panels C and D. (Panel A) Mouse light chain detection in pigs and mice. Lanes: **a**, hybridoma cell line A20/44; **b–e**, DNA from pigs; **b** and **c**, 20 μg and 10 μg DNA from animal 5814; **d**, ear DNA from animal 5814; **e**, ear DNA from a control pig; **f–o**, DNA from tails of mice; **f** and **g**, founder animals 970-08 and 974-10; **h–n**, F1 generation animals derived from 970-08 and 974-10; **o**, DNA from a control mouse. All of the DNAs (panels A and B) were cleaved with *Sac*I. (Panel B) Mouse light chain detection in rabbits. Lanes: **a**, hybridoma cell line A20/44; **b**, control rabbit; **c** and **d**, animals 2644 and 2665 (in lane **d** 1 μg of genomic DNA was used). (Panel C) Detection of the mouse heavy chain in mice and rabbits. Lanes: **a**, control plasmid (0.3 ng) containing the mouse κ and γ-1 gene; **b–k**, DNA from mice; **b** and **c**, founder animals 970-08 and 974-10; **d–j**, DNA from F1 generation animals derived from 970-08 and 974-10; **k**, DNA from a control mouse; **l–n**, DNA from rabbits; **l** and **m**, DNA from animals 2644 and 2665; **n**, DNA from a control rabbit. Each DNA (panels C and D) was cleaved with *Eco*RI + *Hin*dIII. (Panel D) Detection of the mouse heavy chain gene in pigs. Lanes: **a**, hybridoma cell line A20/44; **b**, DNA from a control pig; **c** and **d**, 20 μg and 10 μg DNA from blood cells of animal 5814; **e**, DNA from ear tissue.

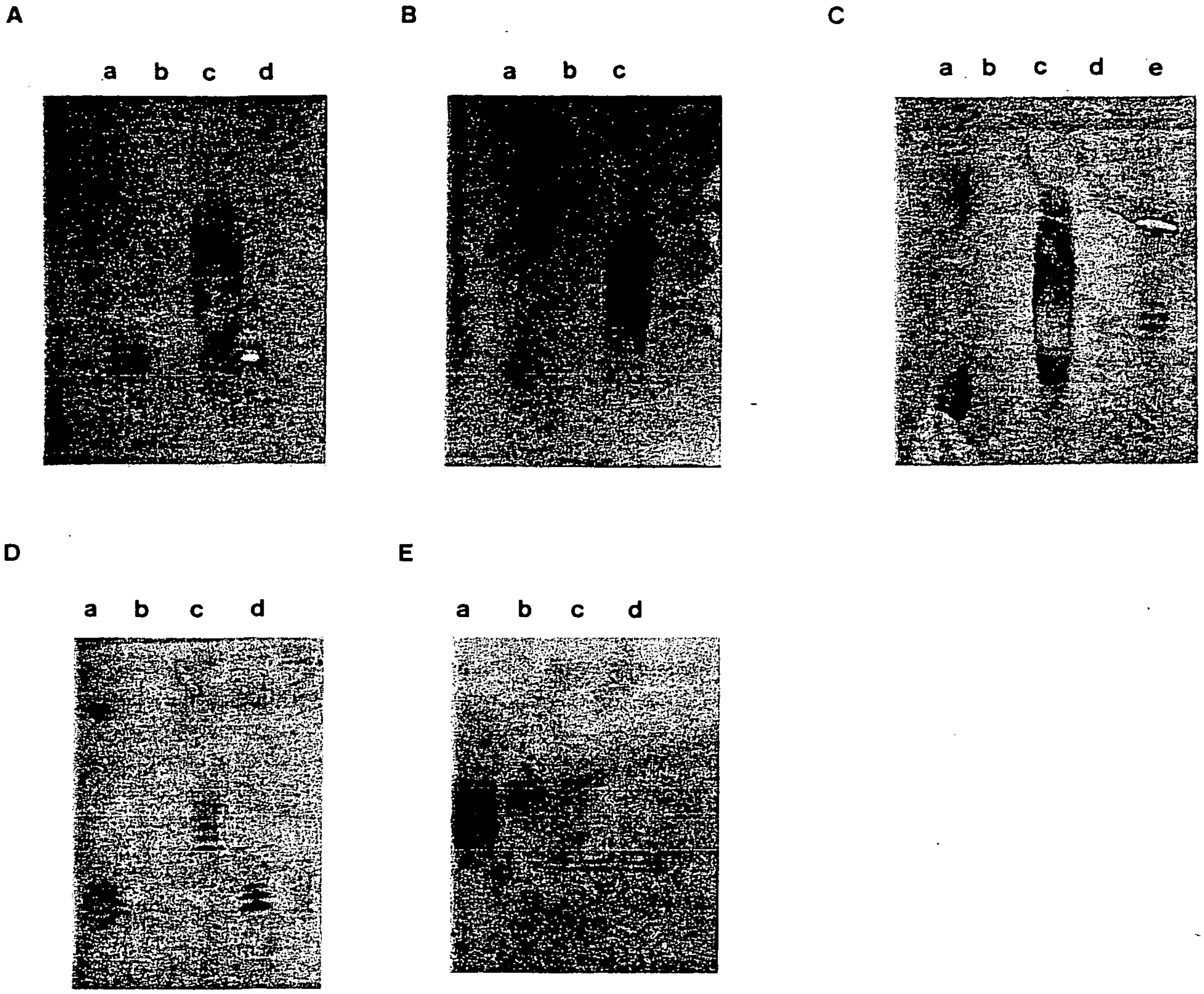

Fig. 4. Analysis of sera and purified Ab by IEF and subsequent immunofixation. Purified Ab or sera were separated by IEF on a phast-gel system (Pharmacia) according to the instructions of the manufacturer. The sera were diluted with 25 mM Tris · HCl pH 7.4/137 mM NaCl/5 mM KCl/0.6 mM Na_2HPO_4. A linear gradient from pH 3 to pH 9 or from pH 5 to pH 8 was established in 5% polyacrylamide gels. Electrophoresis was performed under nondenaturing conditions. The gel was run for 30 min. For immunofixation, the gel was incubated for 45 min with sheep antimouse Fcγ serum (100 μg in a volume of 150 μl) and covered with a cellulose-acetate sheet. The nonreacted proteins were removed by washing with 10 mM Na · phosphate/0.15 M NaCl/0.05% Tween pH 7.2, under shaking overnight. Subsequently, the immunocomplexes were visualised by silver staining (Pharmacia kit). The pH gradient was visualised by calibration proteins. If not otherwise indicated, the pH gradient was from pH 5 to pH 9. mAb20/44 was purified from the serum of transgenic pig No. 5814 by $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-Sepharose and the idiotypic absorbens (MAK43 coupled to Spherosil). Finally, the absorbed fraction was eluted with NP-lysine. Aliquots of the serum and the purified preparation were subjected to IEF analysis and subsequent immunofixation with sheep anti-mouse Fcγ serum and silver staining. (Panels A and B) Analysis of sera from transgenic and control pigs; (panels C and D) analysis of sera from transgenic and control rabbits; (panel E) analysis of crude and purified Ab fractions. Panel A, lanes: **a**, 20 ng Ab A20/44; **b**, control pig (dilution 1 : 20); **c**, from pig 5814 (dilution 1 : 20); **d**, control pig (dilution 1 : 20). Panel B, lanes: **a**, 20 ng Ab A20/44; **b**, control pig (dilution 1 : 50); **c**, pig 5814 (dilution 1 : 50). Panel C, lanes: **a**, Ab A20/44; **b**, control rabbit (dilution 1 : 20); **c**, 1 : 20 dilution from rabbit 2644, **d**, 1 : 1000 dilution of serum from control rabbit; **e**, 1 : 1000 dilutions from rabbit 2644. Panel D, lanes: **a** and **d**, 20 ng of Ab A20/44; **b**, 1 : 1000 dilution of control rabbit; **c**, 1 : 1000 dilution from animal 2665. Panel E, lanes: **a**, crude serum of transgenic pig 5814, **b**, purified preparation; **c** and **d**, MAK 20/44 purified from ascites fluid.

in our constructs the far downstream light-chain enhancer element recently described by Meyer and Neuberger (1989). The differences in IEF patterns could also be attributed to species- and cell-type-specific glycosylation of the heavy chain and possible modification, such as deamidation. This topic is subject of further investigations in our laboratory. In this context it is interesting to note that Möllering et al. (1990) have observed distinct differences in IEF patterns of a mouse mAb from ascites, serum-containing and serum-free media. Also subtle variations in tryptic mapping

profiles were noted. Furthermore, the effect of the transgene on the expression of endogenous heavy and light chains as well as the question whether lymphoid cells are expressing both transgenic and endogenously encoded Ab has to be investigated in detail.

Expression of Ab in the serum of animals could help to protect against diseases (e.g., influenza in pigs).

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